WO0120037

Publication Title:

PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR ALPHA AND DISORDERS OF LIPID METABOLISM

Abstract:

Abstract of WO0120037

The complete genomic structure of the peroxisome proliferator-activated receptor alpha gene is described. Also described are the identification of novel single nucleotide polymorphisms and association with higher plasma LDL-cholesterol and total and LDL-apolipoprotein B concentrations. Data supplied from the esp@cenet database - Worldwide

----

Courtesy of http://v3.espacenet.com

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

PCT

(43) International Publication Date
22 March 2001 (22.03.2001)

(10) International Publication Number
WO 01/20037 A2

(54) Title: PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR ALPHA AND DISORDERS OF LIPID METABOLISM

(57) Abstract: The complete genomic structure of the peroxisome proliferator-activated receptor alpha gene is described. Also
described are the identification of novel single nucleotide polymorphisms and association with higher plasma LDL-cholesterol and
total and LDL-apolipoprotein B concentrations.

PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR ALPHA
AND DISORDERS OF LIPID METABOLISM

RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional application Serial No.
5   60/154,736, filed September 17, 1999, the entire teachings of which are incorporated
herein by reference.

BACKGROUND OF THE INVENTION

Peroxisome proliferator-activated receptors (PPARs) are members of a large
family of ligand-inducible transcription factors that includes receptor for retinoids
10   and vitamin D as well as thyroid and steroid hormones (1). PPARs regulate the
expression of target genes by binding to DNA sequence elements, termed PPAR
response elements (PPRE) (2,3). PPREs have been identified in the regulatory
regions of a variety of genes involved in lipid and energy balance. Three different
PPAR genes have been identified (α, δ and γ) (2,3). PPARγ, expressed mainly in
15   adipose tissue, regulates adipocyte differentiation (4,5). PPARα, the first to be
identified, is expressed mainly in tissues that have a high level of fatty acid (FA)
catabolism such as the liver, kidney, heart and muscle (6,7). Numerous studies have
demonstrated that several genes encoding enzymes involved in β and ω-oxidation
are under the control of PPARα (2,3). PPARα is activated by medium and very
20   long chain FA and polyunsaturated FA, such as eicosapentaenoic acid (8). In
addition to its role in FA oxidation, PPARα appears to play an important role in the
control of extracellular lipid metabolism. In fact, a variety of hypolipidemic fibrates
are synthetic ligands for PPARα (9).

FAs are essential biological components and are used as metabolic fuels,
25   covalent regulators of signaling molecules, and essential components of cellular
membranes. Thus, FA must be kept within narrow physiological limits. Altered
levels of FA are linked to a variety of metabolic diseases including atherosclerosis,
hyperlipidemia, obesity, insulin resistance and type 2 diabetes (10, 11).

-2-

SUMMARY OF THE INVENTION

Peroxisome proliferator-activated receptor alpha (PPARα) is a member of the steroid hormone receptor super family that is involved in the control of cellular lipid utilization. As described herein, the genomic structure of the human PPARα

5   was determined, and intronic primers were designed to sequence the complete coding region and the exon-intron boundaries of the human PPARα gene among 12 patients with type 2 diabetes and 2 controls. Sequence analyses revealed the presence of a L162V missense mutation in exon 5; that is, there is a C to G transversion in the first position of codon 162 leading to a substitution of a valine for

10   the leucine at that position. Mutation L162V is contained within the DNA binding domain of the human PPARα gene, and leucine 162 is highly conserved among humans, mice, rats and guinea pigs. Among diabetics and controls, a trend was observed toward higher plasma LDL-cholesterol and apolipoprotein B levels among carriers of the rare V162 allele. To verify these findings, an independent cohort of

15   193 non-diabetic subjects recruited in the greater Quebec City area was screened. Comparison of the lipoprotein-lipid profile between L162 homozygotes and carriers of the rare V162 allele showed significant differences in plasma LDL-cholesterol, total and LDL-apolipoprotein B concentrations, carriers of the V162 allele having the highest levels. These results suggest that the rare V162 allele may make the

20   subjects more prone to develop a hyperapobetalipoproteinemia.

Thus, the invention relates to the SNPs identified as described herein, both singly and in combination, as well as to the use of these SNPs, and others in PPAR (e.g., PPARα) genes, particularly those nearby in linkage disequilibrium with these SNPs, for diagnosis, prediction of clinical course and treatment response for

25   disorders of lipid metabolism, development of new treatments for disorders of lipid metabolism based upon comparison of the variant and normal versions of the gene or gene product, and development of cell-culture based and animal models for research and treatment of disorders of lipid metabolism. The invention also relates to methods for diagnosing and treating disorders of lipid metabolism, especially high

30   LDL cholesterol levels and hyperapobetalipoproteinemias, and to methods for identifying compounds for use in the diagnosis and treatment of said disorders. The

invention further relates to novel compounds and pharmaceutical compositions for use in the diagnosis and treatment of disorders of lipid metabolism.

The invention relates to isolated nucleic acid molecules comprising all or a portion of the variant allele of PPARα (e.g., wherein reference or wildtype PPARα

5   is exemplified by SEQ ID NO: 1). Preferred portions are at least 10 contiguous nucleotides and comprise the polymorphic site, e.g., a portion of SEQ ID NO: 1 which is at least 10 contiguous nucleotides and comprises the "G" at the first position of codon 162 (nucleotide 696) of the PPARα gene, or a portion of SEQ ID NO: 1 which is at least 10 contiguous nucleotides and comprises the "A" at the last

10   position of codon 253 (nucleotide 971) of the PPARα gene. The invention further relates to isolated gene products, e.g., polypeptides or proteins, which are encoded by a nucleic acid molecule comprising all or a portion of the variant allele of PPARα.

The invention further relates to isolated proteins or polypeptides comprising

15   all or a portion of the variant amino acid sequence of PPARα (e.g., wherein reference or wildtype PPARα is exemplified by SEQ ID NO: 2), and to isolated proteins or polypeptides comprising all or a portion of the variant amino acid sequence of PPARα. Preferred polypeptides are at least 10 contiguous amino acids and comprise the polymorphic amino acid, e.g., a portion of SEQ ID NO: 2 which is

20   at least 10 contiguous amino acids and comprises the valine at residue 162. The invention further relates to isolated nucleic acid molecules encoding such proteins and polypeptides, as well as to antibodies which bind, e.g., specifically, to such proteins and polypeptides.

In one embodiment, the invention relates to a method for predicting the

25   likelihood that an individual will have a disorder of lipid metabolism (or diagnosing or aiding in the diagnosis of a disorder of lipid metabolism), e.g., high LDL cholesterol levels, atherosclerosis, coronary heart disease and/or hyperapobetalipoproteinemias, comprising the steps of obtaining a DNA sample from an individual to be assessed and determining the nucleotide present at the first

30   position of codon 162 (nucleotide 696) of the PPARα gene. The presence of a "C" (the reference nucleotide) at this position indicates that the individual has a lower likelihood of having a disorder of lipid metabolism than an individual having a "G"

-4-

at that position, or a greater likelihood of having less severe symptomology. In a preferred embodiment, the disorder is high LDL cholesterol levels, atherosclerosis and/or coronary heart disease. In another embodiment, the disorder is a hyperapobetalipoproteinemia. In a particular embodiment, the individual is an

5   individual at risk for development of a disorder of lipid metabolism.

In another embodiment, the invention relates to a method for predicting the likelihood that an individual will have a disorder of lipid metabolism (or diagnosing or aiding in the diagnosis of a disorder of lipid metabolism), e.g., high LDL cholesterol levels, atherosclerosis, coronary heart disease and/or

10   hyperapobetalipoproteinemias, comprising the steps of obtaining a DNA sample from an individual to be assessed and determining the nucleotide present at the first position of codon 162 (nucleotide 696) of the PPARα gene. The presence of a "G" (the variant nucleotide) at this position indicates that the individual has a greater likelihood of having a disorder of lipid metabolism than an individual having a "C"

15   at that position, or a greater likelihood of having more severe symptomology. In a preferred embodiment, the disorder is high LDL cholesterol levels, atherosclerosis and/or coronary heart disease. In another embodiment, the disorder is a hyperapobetalipoproteinemia. In a particular embodiment, the individual is an individual at risk for development of a disorder of lipid metabolism.

20   In another embodiment, the invention relates to a method for predicting the likelihood that an individual will have a disorder associated with a "G" at nucleotide position 696 of the PPARα gene (or diagnosing or aiding in the diagnosis of such a disorder) comprising the steps of obtaining a DNA sample from an individual to be assessed and determining the nucleotide present at position 696 of the PPARα gene.

25   The presence of a "G" at this position indicates that the individual has a greater likelihood of having a disorder associated therewith than an individual having a "C" at that position, or a greater likelihood of having more severe symptomology. Conversely, the presence of a "C" at this position indicates that the individual has a lower likelihood of having a disorder associated therewith than an individual having

30   a "G" at that position, or a greater likelihood of having less severe symptomology. In a preferred embodiment, the disorder is high LDL cholesterol levels, atherosclerosis and/or coronary heart disease. In another embodiment, the disorder is a

hyperapobetalipoproteinemia. In a particular embodiment, the individual is an individual at risk for development of such a disorder.

In another embodiment, the invention relates to a method for predicting the likelihood that an individual will have a disorder associated with a "C" at nucleotide

5   position 696 of the PPARα gene (or diagnosing or aiding in the diagnosis of such a disorder) comprising the steps of obtaining a DNA sample from an individual to be assessed and determining the nucleotide present at position 696 of the PPARα gene. The presence of a "C" at this position indicates that the individual has a greater likelihood of having a disorder associated therewith than an individual having a "G"

10   at that position, or a greater likelihood of having more severe symptomology. . Conversely, the presence of a "G" at this position indicates that the individual has a lower likelihood of having a disorder associated therewith than an individual having a "G" at that position, or a greater likelihood of having less severe symptomology. In a preferred embodiment, the disorder is high LDL cholesterol levels,

15   atherosclerosis and/or coronary heart disease. In another embodiment, the disorder is a hyperapobetalipoproteinemia. In a particular embodiment, the individual is an individual at risk for development of such a disorder.

In another embodiment, the invention relates to a method for predicting the likelihood that an individual will have a disorder associated with an "A" at

20   nucleotide position 971 of the PPARα gene (or diagnosing or aiding in the diagnosis of such a disorder) comprising the steps of obtaining a DNA sample from an individual to be assessed and determining the nucleotide present at position 971 of the PPARα gene. The presence of an "A" at this position indicates that the individual has a greater likelihood of having a disorder associated therewith than an

25   individual having a "G" at that position, or a greater likelihood of having more severe symptomology. Conversely, the presence of a "G" at this position indicates that the individual has a lower likelihood of having a disorder associated therewith than an individual having a "A" at that position, or a greater likelihood of having less severe symptomology. In a particular embodiment, the individual is an

30   individual at risk for development of such a disorder.

In another embodiment, the invention relates to a method for predicting the likelihood that an individual will have a disorder associated with a "G" at nucleotide

position 971 of the PPARα gene (or diagnosing or aiding in the diagnosis of such a disorder) comprising the steps of obtaining a DNA sample from an individual to be assessed and determining the nucleotide present at position 971 of the PPARα gene. The presence of a "G" at this position indicates that the individual has a greater

5   likelihood of having a disorder associated therewith than an individual having a "A" at that position, or a greater likelihood of having more severe symptomology. Conversely, the presence of an "A" at this position indicates that the individual has a lower likelihood of having a disorder associated therewith than an individual having a "G" at that position, or a greater likelihood of having less severe symptomology.

10   In a particular embodiment, the individual is an individual at risk for development of such a disorder.

In one embodiment, the invention relates to a method for predicting the likelihood that an individual will have a disorder of lipid metabolism (or diagnosing or aiding in the diagnosis of a disorder of lipid metabolism), e.g., high LDL

15   cholesterol levels, atherosclerosis, coronary heart disease and/or hyperapobetalipoproteinemias, comprising the steps of obtaining a biological sample comprising the PPARα protein or relevant portion thereof from an individual to be assessed and determining the amino acid present at amino acid position 162 of the PPARα protein. The presence of a leucine (the reference amino acid) at this

20   position indicates that the individual has a lower likelihood of having a disorder of lipid metabolism than an individual having a valine at that position, or a greater likelihood of having less severe symptomology. In a preferred embodiment, the disorder is high LDL cholesterol levels, atherosclerosis and/or coronary heart disease. In another embodiment, the disorder is a hyperapobetalipoproteinemia. In

25   a particular embodiment, the individual is an individual at risk for development of a disorder of lipid metabolism.

In another embodiment, the invention relates to a method for predicting the likelihood that an individual will have a disorder of lipid metabolism (or diagnosing or aiding in the diagnosis of a disorder of lipid metabolism), e.g., high LDL

30   cholesterol levels, atherosclerosis, coronary heart disease and/or hyperapobetalipoproteinemias, comprising the steps of obtaining a.biological sample comprising the PPARα protein or relevant portion thereof from an individual to be

assessed and determining the amino acid present at amino acid position 162 of the PPARα protein. The presence of a valine (the variant amino acid) at this position indicates that the individual has a greater likelihood of having a disorder of lipid metabolism than an individual having a leucine at that position, or a greater

5   likelihood of having more severe symptomology. In a preferred embodiment, the disorder is high LDL cholesterol levels, atherosclerosis and/or coronary heart disease. In another embodiment, the disorder is a hyperapobetalipoproteinemia. In a particular embodiment, the individual is an individual at risk for development of a disorder of lipid metabolism.

10   In another embodiment, the invention relates to a method for predicting the likelihood that an individual will have a disorder associated with a valine at amino acid position 162 of the PPARα protein (or diagnosing or aiding in the diagnosis of such a disorder) comprising the steps of obtaining a biological sample comprising the PPARα protein or relevant portion thereof from an individual to be assessed and

15   determining the amino acid present at amino acid position 162 of the PPARα protein. The presence of a valine at this position indicates that the individual has a greater likelihood of having a disorder associated therewith than an individual having a leucine at that position, or a greater likelihood of having more severe symptomology. Conversely, the presence of a leucine at this position indicates that

20   the individual has a lower likelihood of having a disorder associated therewith than an individual having a valine at that position, or a greater likelihood of having less severe symptomology. In a preferred embodiment, the disorder is high LDL cholesterol levels, atherosclerosis and/or coronary heart disease. In another embodiment, the disorder is a hyperapobetalipoproteinemia. In a particular

25   embodiment, the individual is an individual at risk for development of such a disorder.

In another embodiment, the invention relates to a method for predicting the likelihood that an individual will have a disorder associated with a leucine at amino acid position 162 of the PPARα protein (or diagnosing or aiding in the diagnosis of

30   such a disorder) comprising the steps of obtaining a biological sample comprising the PPARα protein or relevant portion thereof from an individual to be assessed and determining the amino acid present at amino acid position 162 of the PPARα

-8-

protein. The presence of a leucine at this position indicates that the individual has a greater likelihood of having a disorder associated therewith than an individual having a valine at that position, or a greater likelihood of having more severe symptomology. Conversely, the presence of a valine at this position indicates that

5 the individual has a lower likelihood of having a disorder associated therewith than an individual having a leucine at that position, or a greater likelihood of having less severe symptomology. In a preferred embodiment, the disorder is high LDL cholesterol levels, atherosclerosis and/or coronary heart disease. In another embodiment, the disorder is a hyperapobetalipoproteinemia. In a particular

10 embodiment, the individual is an individual at risk for development of such a disorder.

In another embodiment, the invention relates to pharmaceutical compositions comprising a reference or variant PPARα gene or gene product, or biologically active portion thereof. The invention relates to pharmaceutical compositions

15 comprising a reference PPARα gene or gene product, or biologically active portion thereof, for use in the treatment of lipid metabolism disorders. The invention further relates to the use of compositions (i.e., agonists and antagonists) which enhance or increase or which reduce or decrease, respectively, the activity of a PPARα gene product for use in the treatment of disorders of lipid metabolism.

20 BRIEF DESCRIPTION OF THE DRAWING

Figure 1 shows a schematic representation of the human PPARα gene. The upper portion is a representation of the human PPARα messenger RNA. The coding region is shaded and arrows indicate initiator and stop codons. The spatial localization of exons within the gene is shown in the lower portion. Intron sizes are

25 also indicated. The gene spans 83.7 Kb of genomic DNA.

Figure 2 shows a nucleotide sequence (SEQ ID NO: 1) of the PPARα mRNA.

Figure 3 shows an amino acid sequence (SEQ ID NO: 2) of the PPARα protein.

DETAILED DESCRIPTION OF THE INVENTION

A list of references referred to herein by number can be found at the end of the Specification.

The human PPARα gene has been mapped on chromosome 22 slightly
5   telomeric to a linkage group of six genes and genetic markers located in the region 22q12-q13.1 (24). As described herein, the structure of the human PPARα gene has been determined. This will facilitate genetics studies evaluating the role of this gene in insulin resistance, type 2 diabetes and dyslipidemia. The human gene spans about 83.7 Kb and is composed of 8 exons. The 5' donor and 3' acceptor splice sites were
10 found to be in agreement to the consensus splice donor and acceptor sequences. The position of introns separating coding exons is conserved between the human and mouse genes (25). However, the sequence of the non-coding portion of the gene shows no significant homology across the two species (24,25). It has been observed that the expression of PPARα mRNA in liver is significantly lower in humans
15 (26,27).

As described herein, direct sequencing of the entire coding region of the human PPARα gene as well as exon-intron boundaries revealed the presence of a silent G to A substitution in exon 7 (nucleotide 971 of the PPARα gene). This substitution does not contribute to an amino acid change, as both polymorphic forms
20 encode a threonine at amino acid position 253, but is considered part of the subject application; for example, this polymorphism can be used to genotype PPARα or in a diagnostic method for disorders associated with one or the other allele. Sequence analyses also revealed the presence of a L162V missense mutation (polymorphism) in exon 5; that is, there is a C to G transversion in the first position of codon 162
25 (nucleotide 696), leading to a substitution of a valine for a leucine at amino acid 162. In this instance, "C" is considered the reference nucleotide and "G" is considered the variant nucleotide.

As used herein, polymorphism refers to the occurrence of two or more genetically determined alternative sequences or alleles in a population. A
30 polymorphic marker or site is the locus at which divergence occurs. Preferred markers have at least two alleles, each occurring at frequency of greater than 1%, and more preferably greater than 10% or 20% of a selected population. A

polymorphic locus may be as small as one base pair, in which case it is referred to as a single nucleotide polymorphism.

The contribution of the L162V missense mutation to the modulation of the lipoprotein-lipid profile was examined. A trend toward higher LDL-cholesterol and
5 plasma total-apolipoprotein B levels was seen among carriers of the V162 allele of the diabetes case-control cohort. The use of a Quebec City non-diabetic male cohort confirmed this tendency, as carriers of the V162 allele had the highest LDL-cholesterol, total and LDL-apolipoprotein B levels. The presence of the V162 allele was associated with a 10.6, 10.3 and 11.7% increase in plasma LDL-cholesterol,
10 total and LDL-apolipoprotein B levels, respectively. These results suggest that the PPARα L162V missense mutation may modulate the concentration of apolipoprotein B-containing lipoproteins. Thus, carriers of the rare V162 allele may be more prone to develop atherosclerosis and subsequent coronary heart disease, since increased levels of LDL-cholesterol and apolipoprotein B levels are well
15 known risk factors for heart disease (28-31).

Thus, it is possible that changes in PPARα activity could contribute to the observed lipoprotein-lipid variability in the population. Results presented herein suggest that the L162V mutation may modulate plasma lipoprotein-lipid levels. This missense mutation results in a non-conservative amino acid substitution located
20 within the DNA binding domain or C domain of the protein (26). This domain, which targets the receptor to specific DNA sequences known as hormone response element (HRE) or PPRE for PPAR specific genes, is the most conserved among nuclear receptor domains, (2,3,26). Taken together, these observations suggest that the leucine 162 may be important for the activity of PPARα and thus the valine
25 substitution may alter its activity. It is also possible that the L162V mutation is in linkage disequilibrium with a functional mutation in another gene in the neighborhood that regulates blood lipid concentrations.

The invention relates to isolated nucleic acid molecules comprising all or a portion of the variant allele of PPARα (e.g., wherein reference or wildtype PPARα
30 is exemplified by SEQ ID NO: 1). Preferred portions are at least 10 contiguous nucleotides and comprise the polymorphic site, e.g., a portion of SEQ ID NO: 1 which is at least 10 contiguous nucleotides and comprises the "G" at the first

position of codon 162 (nucleotide 696) of the PPARα gene, or a portion of SEQ ID NO: 1 which is at least 10 contiguous nucleotides and comprises the "A" at the last position of codon 253 (nucleotide 971) of the PPARα gene. The invention further relates to isolated gene products, e.g., polypeptides or proteins, which are encoded

5   by a nucleic acid molecule comprising all or a portion of the variant allele of PPARα.

A nucleic acid molecule or oligonucleotide can be DNA or RNA, and single- or double-stranded. Nucleic acid molecules and oligonucleotides can be naturally occurring or synthetic, but are typically prepared by synthetic means. Preferred

10   nucleic acid molecules and oligonucleotides of the invention include segments of DNA, or their complements, which include any one of the polymorphic sites shown in the Table. The segments can be between 5 and 250 bases, and, in specific embodiments, are between 5-10, 5-20, 10-20, 10-50, 20-50 or 10-100 bases. For example, the segment can be 21 bases. The polymorphic site can occur within any

15   position of the segment.

The invention also relates to nucleic acid molecules which hybridize to the variant alleles identified herein (or their complements) and which also comprise the variant nucleotide at the SNP site. Hybridizations are usually performed under stringent conditions, for example, at a salt concentration of no more than 1 M and a

20   temperature of at least 25°C. For example, conditions of 5X SSPE (750 mM NaCl, 50 mM NaPhosphate, 5 mM EDTA, pH 7.4) and a temperature of 25-30°C, or equivalent conditions, are suitable for allele-specific probe hybridizations. Equivalent conditions can be determined by varying one or more of the parameters given as an example, as known in the art, while maintaining a similar degree of

25   identity or similarity between the target nucleotide sequence and the primer or probe used.

The invention also relates to nucleic acid molecules which share substantial sequence identity to the variant alleles identified herein (or their complements) and which also comprise the variant nucleotide at the SNP site. Particularly preferred

30   are nucleic acid molecules and fragments which have at least about 60%, preferably at least about 70, 80 or 85%, more preferably at least about 90%, even more preferably at least about 95%, and most preferably at least about 98% identity with

-12-

nucleic acid molecules described herein. The percent identity of two nucleotide or amino acid sequences can be determined by aligning the sequences for optimal comparison purposes (*e.g.*, gaps can be introduced in the sequence of a first sequence). The nucleotides or amino acids at corresponding positions arc then

5   compared, and the percent identity between the two sequences is a function of the number of identical positions shared by the sequences (*i.e.*, % identity = # of identical positions/total # of positions x 100). In certain embodiments, the length of a sequence aligned for comparison purposes is at least 30%, preferably at least 40%, more preferably at least 60%, and even more preferably at least 70%, 80% or 90% of

10   the length of the reference sequence. The actual comparison of the two sequences can be accomplished by well-known methods, for example, using a mathematical algorithm. A preferred, non-limiting example of such a mathematical algorithm is described in Karlin *et al.*, *Proc. Natl. Acad. Sci. USA, 90*:5873-5877 (1993). Such an algorithm is incorporated into the NBLAST and XBLAST programs (version 2.0)

15   as described in Altschul *et al.*, *Nucleic Acids Res., 25*:389-3402 (1997). When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective programs (*e.g.*, NBLAST) can be used. See http://www.ncbi.nlm.nih.gov. In one embodiment, parameters for sequence comparison can be set at score=100, wordlength=12, or can be varied (*e.g.*, W=5 or

20   W=20).

The term "isolated" is used herein to indicate that the material in question exists in a physical milieu distinct from that in which it occurs in nature. For example, an isolated nucleic acid of the invention may be substantially isolated with respect to the complex cellular milieu in which it naturally occurs. In some

25   instances, the isolated material will form part of a composition (for example, a crude extract containing other substances), buffer system or reagent mix. In other circumstance, the material may be purified to essential homogeneity, for example as determined by PAGE or column chromatography such as HPLC. Preferably, an isolated nucleic acid comprises at least about 50, 80 or 90 percent (on a molar basis)

30   of all macromolecular species present.

Thus, the invention relates to a method for predicting the likelihood that an individual will have a disorder of lipid metabolism (or diagnosing or aiding in the

diagnosis of a disorder of lipid metabolism), e.g., high LDL cholesterol levels, atherosclerosis, coronary heart disease and/or hyperapobetalipoproteinemias, comprising the steps of obtaining a DNA sample from an individual to be assessed and determining the nucleotide present at the first position of codon 162 (nucleotide

5   696) of the PPARα gene. The presence of a "C" (the reference nucleotide) at this position indicates that the individual has a lower likelihood of having a disorder of lipid metabolism than an individual having a "G" at that position, or a greater likelihood of having less severe symptomology. In a preferred embodiment, the disorder is high LDL cholesterol levels, atherosclerosis and/or coronary heart

10   disease. In another embodiment, the disorder is a hyperapobetalipoproteinemia. In a particular embodiment, the individual is an individual at risk for development of a disorder of lipid metabolism.

In another embodiment, the invention relates to a method for predicting the likelihood that an individual will have a disorder of lipid metabolism (or diagnosing

15   or aiding in the diagnosis of a disorder of lipid metabolism), e.g., high LDL cholesterol levels, atherosclerosis, coronary heart disease and/or hyperapobetalipoproteinemias, comprising the steps of obtaining a DNA sample from an individual to be assessed and determining the nucleotide present at the first position of codon 162 (nucleotide 696) of the PPARα gene. The presence of a "G"

20   (the variant nucleotide) at this position indicates that the individual has a greater likelihood of having a disorder of lipid metabolism than an individual having a "C" at that position, or a greater likelihood of having more severe symptomology. In a preferred embodiment, the disorder is high LDL cholesterol levels, atherosclerosis and/or coronary heart disease. In another embodiment, the disorder is a

25   hyperapobetalipoproteinemia. In a particular embodiment, the individual is an individual at risk for development of a disorder of lipid metabolism.

As used herein, disorders of lipid metabolism include, but are not limited to, abnormal lipoprotein-lipid variability, abnormal LDL-cholesterol levels, abnormal plasma total-apolipoprotein B levels, atherosclerosis and coronary heart disease. In

30   a particular embodiment, the individual is an individual at risk for development of disorders of lipid metabolism. In another embodiment the individual exhibits clinical symptomology associated with disorders of lipid metabolism. In one

-14-

embodiment, the individual has been clinically diagnosed as having one or more disorders of lipid metabolism.

In another embodiment, the invention relates to a method for predicting the likelihood that an individual will have a disorder associated with a "G" at nucleotide

5   position 696 of the PPARα gene (or diagnosing or aiding in the diagnosis of such a disorder) comprising the steps of obtaining a DNA sample from an individual to be assessed and determining the nucleotide present at position 696 of the PPARα gene. The presence of a "G" at this position indicates that the individual has a greater likelihood of having a disorder associated therewith than an individual having a "C"

10 at that position, or a greater likelihood of having more severe symptomology. Conversely, the presence of a "C" at this position indicates that the individual has a lower likelihood of having a disorder associated therewith than an individual having a "G" at that position, or a greater likelihood of having less severe symptomology. In a preferred embodiment, the disorder is high LDL cholesterol levels, atherosclerosis

15 and/or coronary heart disease. In another embodiment, the disorder is a hyperapobetalipoproteinemia. In a particular embodiment, the individual is an individual at risk for development of such a disorder.

In another embodiment, the invention relates to a method for predicting the likelihood that an individual will have a disorder associated with a "C" at nucleotide

20 position 696 of the PPARα gene (or diagnosing or aiding in the diagnosis of such a disorder) comprising the steps of obtaining a DNA sample from an individual to be assessed and determining the nucleotide present at position 696 of the PPARα gene. The presence of a "C" at this position indicates that the individual has a greater likelihood of having a disorder associated therewith than an individual having a "G"

25 at that position, or a greater likelihood of having more severe symptomology. Conversely, the presence of a "G" at this position indicates that the individual has a lower likelihood of having a disorder associated therewith than an individual having a "G" at that position, or a greater likelihood of having less severe symptomology. In a preferred embodiment, the disorder is high LDL cholesterol levels,

30 atherosclerosis and/or coronary heart disease. In another embodiment, the disorder is a hyperapobetalipoproteinemia. In a particular embodiment, the individual is an individual at risk for development of such a disorder.

-15-

In another embodiment, the invention relates to a method for predicting the likelihood that an individual will have a disorder associated with an "A" at nucleotide position 971 of the PPARα gene (or diagnosing or aiding in the diagnosis of such a disorder) comprising the steps of obtaining a DNA sample from an

5   individual to be assessed and determining the nucleotide present at position 971 of the PPARα gene. The presence of an "A" at this position indicates that the individual has a greater likelihood of having a disorder associated therewith than an individual having a "G" at that position, or a greater likelihood of having more severe symptomology. Conversely, the presence of a "G" at this position indicates

10   that the individual has a lower likelihood of having a disorder associated therewith than an individual having a "A" at that position, or a greater likelihood of having less severe symptomology. In a particular embodiment, the individual is an individual at risk for development of such a disorder.

In another embodiment, the invention relates to a method for predicting the

15   likelihood that an individual will have a disorder associated with a "G" at nucleotide position 971 of the PPARα gene (or diagnosing or aiding in the diagnosis of such a disorder) comprising the steps of obtaining a DNA sample from an individual to be assessed and determining the nucleotide present at position 971 of the PPARα gene. The presence of a "G" at this position indicates that the individual has a greater

20   likelihood of having a disorder associated therewith than an individual having a "A" at that position, or a greater likelihood of having more severe symptomology. Conversely, the presence of an "A" at this position indicates that the individual has a lower likelihood of having a disorder associated therewith than an individual having a "G" at that position, or a greater likelihood of having less severe symptomology.

25   In a particular embodiment, the individual is an individual at risk for development of such a disorder.

The genetic material to be assessed can be obtained from any nucleated cell from the individual. For assay of genomic DNA, virtually any biological sample (other than pure red blood cells) is suitable. For example, convenient tissue samples

30   include whole blood, semen, saliva, tears, urine, fecal material, sweat, skin and hair. For assay of cDNA or mRNA, the tissue sample must be obtained from an organ in which the target nucleic acid is expressed. For example, cells from tissues that have

a relatively high level of fatty acid catabolism (such as cells of the liver, kidney, heart and muscle) are suitable sources for obtaining cDNA for the PPARα gene.

Many of the methods described herein require amplification of DNA from target samples. This can be accomplished by e.g., PCR. *See generally PCR*

5   *Technology: Principles and Applications for DNA Amplification* (ed. H.A. Erlich, Freeman Press, NY, NY, 1992); *PCR Protocols: A Guide to Methods and Applications* (eds. Innis, *et al.*, Academic Press, San Diego, CA, 1990); Mattila *et al., Nucleic Acids Res.* 19, 4967 (1991); Eckert *et al., PCR Methods and Applications* 1, 17 (1991); *PCR* (eds. McPherson *et al.*, IRL Press, Oxford); and

10   U.S. Patent 4,683,202.

Other suitable amplification methods include the ligase chain reaction (LCR) (see Wu and Wallace, *Genomics* 4, 560 (1989), Landegren *et al., Science* 241, 1077 (1988), transcription amplification (Kwoh *et al., Proc. Natl. Acad. Sci. USA* 86, 1173 (1989)), and self-sustained sequence replication (Guatelli *et al., Proc. Nat.*

15   *Acad. Sci. USA,* 87, 1874 (1990)) and nucleic acid based sequence amplification (NASBA). The latter two amplification methods involve isothermal reactions based on isothermal transcription, which produce both single stranded RNA (ssRNA) and double stranded DNA (dsDNA) as the amplification products in a ratio of about 30 or 100 to 1, respectively.

20   The nucleotide which occupies the polymorphic site of interest (e.g., the first nucleotide in codon 162 of PPARα) can be identified by a variety methods, such as Southern analysis of genomic DNA; Northern analysis of RNA; denaturing high pressure liquid chromatography (DHPLC); gene isolation and sequencing; hybridization of an allele-specific oligonucleotide with amplified gene products;

25   single base extension (SBE); or analysis of the PPARα protein. In a preferred embodiment, determination of the allelic form of PPARα is carried out using SBE-FRET methods, or using chip-based oligonucleotide arrays. A sampling of suitable procedures is discussed below in turn.

1. Allele-Specific Probes

30   The design and use of allele-specific probes for analyzing polymorphisms is described by e.g., Saiki *et al., Nature* 324, 163-166 (1986); Dattagupta, EP 235,726,

-17-

Saiki, WO 89/11548. Allele-specific probes can be designed that hybridize to a segment of target DNA from one individual but do not hybridize to the corresponding segment from another individual due to the presence of different polymorphic forms in the respective segments from the two individuals.

5   Hybridization conditions should be sufficiently stringent that there is a significant difference in hybridization intensity between alleles, and preferably an essentially binary response, whereby a probe hybridizes to only one of the alleles. Hybridizations are usually performed under stringent conditions, for example, at a salt concentration of no more than 1 M and a temperature of at least 25°C. For

10   example, conditions of 5X SSPE (750 mM NaCl, 50 mM NaPhosphate, 5 mM EDTA, pH 7.4) and a temperature of 25-30°C, or equivalent conditions, are suitable for allele-specific probe hybridizations. Equivalent conditions can be determined by varying one or more of the parameters given as an example, as known in the art, while maintaining a similar degree of identity or similarity between the target

15   nucleotide sequence and the primer or probe used.

Some probes are designed to hybridize to a segment of target DNA such that the polymorphic site aligns with a central position (e.g., in a 15-mer at the 7 position; in a 16-mer, at either the 8 or 9 position) of the probe. This design of probe achieves good discrimination in hybridization between different allelic forms.

20   Allele-specific probes are often used in pairs, one member of a pair showing a perfect match to a reference form of a target sequence and the other member showing a perfect match to a variant form. Several pairs of probes can then be immobilized on the same support for simultaneous analysis of multiple polymorphisms within the same target sequence.

25   2. Tiling Arrays

The polymorphisms can also be identified by hybridization to nucleic acid arrays, some examples of which are described in WO 95/11995. WO 95/11995 also describes subarrays that are optimized for detection of a variant form of a precharacterized polymorphism. Such a subarray contains probes designed to be

30   complementary to a second reference sequence, which is an allelic variant of the first reference sequence. The second group of probes is designed by the same principles,

-18-

except that the probes exhibit complementarity to the second reference sequence. The inclusion of a second group (or further groups) can be particularly useful for analyzing short subsequences of the primary reference sequence in which multiple mutations are expected to occur within a short distance commensurate with the

5   length of the probes (e.g., two or more mutations within 9 to 21 bases).

       3. Allele-Specific Primers

       An allele-specific primer hybridizes to a site on target DNA overlapping a polymorphism and only primes amplification of an allelic form to which the primer exhibits perfect complementarity. See Gibbs, *Nucleic Acid Res.* 17, 2427-2448

10   (1989). This primer is used in conjunction with a second primer which hybridizes at a distal site. Amplification proceeds from the two primers, resulting in a detectable product which indicates the particular allelic form is present. A control is usually performed with a second pair of primers, one of which shows a single base mismatch at the polymorphic site and the other of which exhibits perfect

15   complementarity to a distal site. The single-base mismatch prevents amplification and no detectable product is formed. The method works best when the mismatch is included in the 3'-most position of the oligonucleotide aligned with the polymorphism because this position is most destabilizing to elongation from the primer (see, e.g., WO 93/22456).

20   4. Direct-Sequencing

       The direct analysis of the sequence of polymorphisms of the present invention can be accomplished using either the dideoxy chain termination method or the Maxam Gilbert method (see Sambrook *et al., Molecular Cloning, A Laboratory Manual* (2nd Ed., CSHP, New York 1989); Zyskind *et al., Recombinant DNA*

25   *Laboratory Manual,* (Acad. Press, 1988)).

       5. Denaturing Gradient Gel Electrophoresis

       Amplification products generated using the polymerase chain reaction can be analyzed by the use of denaturing gradient gel electrophoresis. Different alleles can be identified based on the different sequence-dependent melting properties and

-19-

electrophoretic migration of DNA in solution. Erlich, ed., *PCR Technology, Principles and Applications for DNA Amplification*, (W.H. Freeman and Co, New York, 1992), Chapter 7.

6. Single-Strand Conformation Polymorphism Analysis

Alleles of target sequences can be differentiated using single-strand conformation polymorphism analysis, which identifies base differences by alteration in electrophoretic migration of single stranded PCR products, as described in Orita *et al.*, *Proc. Nat. Acad. Sci.* 86, 2766-2770 (1989). Amplified PCR products can be generated as described above, and heated or otherwise denatured, to form single stranded amplification products. Single-stranded nucleic acids may refold or form secondary structures which are partially dependent on the base sequence. The different electrophoretic mobilities of single-stranded amplification products can be related to base-sequence differences between alleles of target sequences.

7. Single-Base Extension

An alternative method for identifying and analyzing polymorphisms is based on single-base extension (SBE) of a fluorescently-labeled primer coupled with fluorescence resonance energy transfer (FRET) between the label of the added base and the label of the primer. Typically, the method, such as that described by Chen *et al.*, (*PNAS 94*:10756-61 (1997), incorporated herein by reference) uses a locus-specific oligonucleotide primer labeled on the 5' terminus with 5-carboxyfluorescein (FAM). This labeled primer is designed so that the 3' end is immediately adjacent to the polymorphic site of interest. The labeled primer is hybridized to the locus, and single base extension of the labeled primer is performed with fluorescently labeled dideoxyribonucleotides (ddNTPs) in dye-terminator sequencing fashion, except that no deoxyribonucleotides are present. An increase in fluorescence of the added ddNTP in response to excitation at the wavelength of the labeled primer is used to infer the identity of the added nucleotide.

The variant allele described herein may contribute to the bias of an individual toward disorders of lipid metabolism in one or more different ways. The

-20-

polymorphism may contribute to phenotype by affecting protein structure as a result of the alteration in amino acid sequence. The polymorphism may exert phenotypic effects indirectly via influence on replication, transcription, and translation. More than one phenotypic trait may be affected. For example, other disorders which are

5   related to disorders of lipid metabolism may also be affected by the PPARα polymorphism described herein. The polymorphism may also alter the interaction of the gene or gene product with drugs which normally interact directly with PPARα (e.g., fibrates and other drugs which affect lipid metabolism). Additionally, the described polymorphism may predispose an individual to a distinct mutation that is

10   causally related to a certain phenotype, such as susceptibility or resistance to disorders of lipid metabolism. The discovery of the polymorphism and its correlation with disorders of lipid metabolism facilitates biochemical analysis of the variant and the development of assays to characterize the variant and to screen for pharmaceuticals that interact directly with one or another form of the protein.

15   Alternatively, this particular polymorphism may be one of a group of two or more polymorphisms in the PPARα gene which contributes to the presence, absence or severity of the disorder of lipid metabolism. An assessment of other polymorphisms within the gene can be undertaken, and the separate and combined effects of these polymorphisms on the disorder phenotype can be assessed.

20   Correlation between a particular phenotype, e.g., the high LDL-cholesterol phenotype, and the presence or absence of a particular allele is performed for a population of individuals who have been tested for the presence or absence of the phenotype. Correlation can be performed by standard statistical methods such as a Chi-squared test and statistically significant correlations between polymorphic

25   form(s) and phenotypic characteristics are noted.

       This correlation can be exploited in several ways. In the case of a strong correlation between a particular polymorphic form, e.g., the variant allele for PPARα, and a disorder for which treatment is available, detection of the polymorphic form in an individual may justify immediate administration of

30   treatment, or at least the institution of regular monitoring of the individual. Detection of a polymorphic form correlated with a disorder in a couple contemplating a family may also be valuable to the couple in their reproductive

decisions. For example, the female partner might elect to undergo *in vitro* fertilization to avoid the possibility of transmitting such a polymorphism from her husband to her offspring. In the case of a weaker, but still statistically significant correlation between a polymorphic form and a particular disorder, immediate

5   therapeutic intervention or monitoring may not be justified. Nevertheless, the individual can be motivated to begin simple life-style changes (e.g., diet, therapy or counseling) that can be accomplished at little cost to the individual but confer potential benefits in reducing the risk of conditions to which the individual may have increased susceptibility by virtue of the particular allele. Furthermore,

10   identification of a polymorphic form correlated with enhanced receptiveness to one of several treatment regimes for a disorder indicates that this treatment regime should be followed for the individual in question.

Furthermore, it may be possible to identify a physical linkage between a genetic locus associated with a trait of interest and polymorphic markers that are not

15   associated with the trait, but are in physical proximity with the genetic locus responsible for the trait and co-segregate with it. Such analysis is useful for mapping a genetic locus associated with a phenotypic trait to a chromosomal position, and thereby cloning gene(s) responsible for the trait. *See* Lander *et al.*, *Proc. Natl. Acad. Sci. (USA)* 83, 7353-7357 (1986); Lander *et al.*, *Proc. Natl. Acad.*

20   *Sci. (USA)* 84, 2363-2367 (1987); Donis-Keller *et al.*, *Cell* 51, 319-337 (1987); Lander *et al.*, *Genetics* 121, 185-199 (1989)). Genes localized by linkage can be cloned by a process known as directional cloning. *See* Wainwright, *Med. J. Australia* 159, 170-174 (1993); Collins, *Nature Genetics* 1, 3-6 (1992).

Linkage studies are typically performed on members of a family. Available

25   members of the family are characterized for the presence or absence of a phenotypic trait and for a set of polymorphic markers. The distribution of polymorphic markers in an informative meiosis is then analyzed to determine which polymorphic markers co-segregate with a phenotypic trait. *See, e.g.*, Kerem *et al.*, *Science* 245, 1073-1080 (1989); Monaco *et al.*, *Nature* 316, 842 (1985); Yamoka *et al.*, *Neurology* 40, 222-

30   226 (1990); Rossiter *et al.*, *FASEB Journal* 5, 21-27 (1991).

Linkage is analyzed by calculation of LOD (log of the odds) values. A lod value is the relative likelihood of obtaining observed segregation data for a marker

-22-

and a genetic locus when the two are located at a recombination fraction θ, versus the situation in which the two are not linked, and thus segregating independently (Thompson & Thompson, *Genetics in Medicine* (5th ed, W.B. Saunders Company, Philadelphia, 1991); Strachan, "Mapping the human genome" in *The Human*

5   *Genome* (BIOS Scientific Publishers Ltd, Oxford), Chapter 4). A series of likelihood ratios are calculated at various recombination fractions (θ), ranging from $\theta = 0.0$ (coincident loci) to $\theta = 0.50$ (unlinked). Thus, the likelihood at a given value of θ is: probability of data if loci linked at θ to probability of data if loci unlinked. The computed likelihoods are usually expressed as the $\log_{10}$ of this ratio

10   (i.e., a lod score). For example, a lod score of 3 indicates 1000:1 odds against an apparent observed linkage being a coincidence. The use of logarithms allows data collected from different families to be combined by simple addition. Computer programs are available for the calculation of lod scores for differing values of θ (e.g., LIPED, MLINK (Lathrop, *Proc. Nat. Acad. Sci. (USA)* 81, 3443-3446 (1984)).

15   For any particular lod score, a recombination fraction may be determined from mathematical tables. *See* Smith *et al.*, *Mathematical tables for research workers in human genetics* (Churchill, London, 1961); Smith, *Ann. Hum. Genet.* 32, 127-150 (1968). The value of θ at which the lod score is the highest is considered to be the best estimate of the recombination fraction.

20   Positive lod score values suggest that the two loci are linked, whereas negative values suggest that linkage is less likely (at that value of θ) than the possibility that the two loci are unlinked. By convention, a combined lod score of +3 or greater (equivalent to greater than 1000:1 odds in favor of linkage) is considered definitive evidence that two loci are linked. Similarly, by convention, a

25   negative lod score of -2 or less is taken as definitive evidence against linkage of the two loci being compared. Negative linkage data are useful in excluding a chromosome or a segment thereof from consideration. The search focuses on the remaining non-excluded chromosomal locations.

            The invention further relates to isolated proteins or polypeptides comprising

30   all or a portion of the variant amino acid sequence of PPARα (e.g., wherein reference or wildtype PPARα is exemplified by SEQ ID NO: 2; Figure 3), and to isolated proteins or polypeptides comprising all or a portion of the variant amino

acid sequence of PPARα. Preferred polypeptides are at least 10 contiguous amino acids and comprise the polymorphic amino acid, e.g., a portion of SEQ ID NO: 2 which is at least 10 contiguous amino acids and comprises the valine at residue 162. The invention further relates to isolated nucleic acid molecules encoding such

5 proteins and polypeptides. In addition, the present invention includes biologically active fragments of the polypeptides, or analogs thereof, including organic molecules which simulate the interactions of the peptides. Biologically active fragments include any portion of the full-length polypeptide which confers a biological function on the gene product, including ligand binding, and antibody

10 binding. Ligand binding includes binding by nucleic acids, proteins or polypeptides, small biologically active molecules, or large cellular structures.

In one embodiment, the invention relates to a method for predicting the likelihood that an individual will have a disorder of lipid metabolism (or diagnosing or aiding in the diagnosis of a disorder of lipid metabolism), e.g., high LDL

15 cholesterol levels, atherosclerosis, coronary heart disease and/or hyperapobetalipoproteinemias, comprising the steps of obtaining a biological sample comprising the PPARα protein or relevant portion thereof from an individual to be assessed and determining the amino acid present at amino acid position 162 of the PPARα protein. The presence of a leucine (the reference amino acid) at this

20 position indicates that the individual has a lower likelihood of having a disorder of lipid metabolism than an individual having a valine at that position, or a greater likelihood of having less severe symptomology. In a preferred embodiment, the disorder is high LDL cholesterol levels, atherosclerosis and/or coronary heart disease. In another embodiment, the disorder is a hyperapobetalipoproteinemia. In

25 a particular embodiment, the individual is an individual at risk for development of a disorder of lipid metabolism.

In another embodiment, the invention relates to a method for predicting the likelihood that an individual will have a disorder of lipid metabolism (or diagnosing or aiding in the diagnosis of a disorder of lipid metabolism), e.g., high LDL

30 cholesterol levels, atherosclerosis, coronary heart disease and/or hyperapobetalipoproteinemias, comprising the steps of obtaining a.biological sample comprising the PPARα protein or relevant portion thereof from an individual to be

assessed and determining the amino acid present at amino acid position 162 of the PPARα protein. The presence of a valine (the variant amino acid) at this position indicates that the individual has a greater likelihood of having a disorder of lipid metabolism than an individual having a leucine at that position, or a greater

5   likelihood of having more severe symptomology. In a preferred embodiment, the disorder is high LDL cholesterol levels, atherosclerosis and/or coronary heart disease. In another embodiment, the disorder is a hyperapobetalipoproteinemia. In a particular embodiment, the individual is an individual at risk for development of a disorder of lipid metabolism.

10   In another embodiment, the invention relates to a method for predicting the likelihood that an individual will have a disorder associated with a valine at amino acid position 162 of the PPARα protein (or diagnosing or aiding in the diagnosis of such a disorder) comprising the steps of obtaining a biological sample comprising the PPARα protein or relevant portion thereof from an individual to be assessed and

15   determining the amino acid present at amino acid position 162 of the PPARα protein. The presence of a valine at this position indicates that the individual has a greater likelihood of having a disorder associated therewith than an individual having a leucine at that position, or a greater likelihood of having more severe symptomology. Conversely, the presence of a leucine at this position indicates that

20   the individual has a lower likelihood of having a disorder associated therewith than an individual having a valine at that position, or a greater likelihood of having less severe symptomology. In a preferred embodiment, the disorder is high LDL cholesterol levels, atherosclerosis and/or coronary heart disease. In another embodiment, the disorder is a hyperapobetalipoproteinemia. In a particular

25   embodiment, the individual is an individual at risk for development of such a disorder.

In another embodiment, the invention relates to a method for predicting the likelihood that an individual will have a disorder associated with a leucine at amino acid position 162 of the PPARα protein (or diagnosing or aiding in the diagnosis of

30   such a disorder) comprising the steps of obtaining a biological sample comprising the PPARα protein or relevant portion thereof from an individual to be assessed and determining the amino acid present at amino acid position 162 of the PPARα

protein. The presence of a leucine at this position indicates that the individual has a greater likelihood of having a disorder associated therewith than an individual having a valine at that position, or a greater likelihood of having more severe symptomology. Conversely, the presence of a valine at this position indicates that

5 the individual has a lower likelihood of having a disorder associated therewith than an individual having a leucine at that position, or a greater likelihood of having less severe symptomology. In a preferred embodiment, the disorder is high LDL cholesterol levels, atherosclerosis and/or coronary heart disease. In another embodiment, the disorder is a hyperapobetalipoproteinemia. In a particular

10 embodiment, the individual is an individual at risk for development of such a disorder.

In these embodiments of the invention, the biological sample contains protein molecules from the test subject. The term "relevant portion" of the protein is intended to mean that the portion of the protein contains the relevant amino acid

15 position to be analysed. *In vitro* techniques for detection of protein include enzyme linked immunosorbent assays (ELISAs), Western blots, immunoprecipitations and immunofluorescence. Furthermore, *in vivo* techniques for detection of protein include introducing into a subject a labeled anti-protein antibody. For example, the antibody can be labeled with a radioactive marker whose presence and location in a

20 subject can be detected by standard imaging techniques. Polyclonal and/or monoclonal antibodies that specifically bind to variant gene products but not to corresponding reference gene products, and vice versa, are also provided. Antibodies can be made by injecting mice or other animals with the variant gene product or synthetic peptide fragments thereof comprising the variant portion.

25 Monoclonal antibodies are screened as are described, for example, in Harlow & Lane, *Antibodies, A Laboratory Manual*, Cold Spring Harbor Press, New York (1988); Goding, *Monoclonal antibodies, Principles and Practice* (2d ed.) Academic Press, New York (1986). Monoclonal antibodies are tested for specific immunoreactivity with a variant gene product and lack of immunoreactivity to the

30 corresponding prototypical gene product. These antibodies are useful in diagnostic assays for detection of the variant form, or as an active ingredient in a pharmaceutical composition.

-26-

Polyclonal and/or monoclonal antibodies that specifically bind to variant gene products but not to corresponding reference gene products, or to reference gene products and not to corresponding variant gene products, are also provided. Antibodies can be made by injecting mice or other animals with the variant gene

5   product or synthetic peptide fragments thereof comprising the variant portion. Monoclonal antibodies are screened as are described, for example, in Harlow & Lane, *Antibodies, A Laboratory Manual*, Cold Spring Harbor Press, New York (1988); Goding, *Monoclonal antibodies, Principles and Practice* (2d ed.) Academic Press, New York (1986). Monoclonal antibodies are tested for specific

10   immunoreactivity with a variant gene product and lack of immunoreactivity to the corresponding prototypical gene product. These antibodies are useful in diagnostic assays for detection of the variant form, or as an active ingredient in a pharmaceutical composition.

The invention further pertains to compositions, e.g., vectors, comprising a

15   nucleotide sequence encoding the reference or variant gene product. For example, variant genes can be expressed in an expression vector in which a variant gene is operably linked to a native or other promoter. Usually, the promoter is a eukaryotic promoter for expression in a mammalian cell. The transcription regulation sequences typically include a heterologous promoter and optionally an enhancer

20   which is recognized by the host. The selection of an appropriate promoter, for example trp, lac, phage promoters, glycolytic enzyme promoters and tRNA promoters, depends on the host selected. Commercially available expression vectors can be used. Vectors can include host-recognized replication systems, amplifiable genes, selectable markers, host sequences useful for insertion into the host genome,

25   and the like.

The means of introducing the expression construct into a host cell varies depending upon the particular construction and the target host. Suitable means include fusion, conjugation, transfection, transduction, electroporation or injection, as described in Sambrook, *supra*. A wide variety of host cells can be employed for

30   expression of the variant gene, both prokaryotic and eukaryotic. Suitable host cells include bacteria such as *E. coli*, yeast, filamentous fungi, insect cells, mammalian cells, typically immortalized, *e.g.*, mouse, CHO, human and monkey cell lines and

derivatives thereof. Preferred host cells are able to process the variant gene product to produce an appropriate mature polypeptide. Processing includes glycosylation, ubiquitination, disulfide bond formation, general post-translational modification, and the like.

5   It is also contemplated that cells can be engineered to express the variant or reference allele of the invention by gene therapy methods. For example, DNA encoding the reference PPARα gene product, or an active fragment or derivative thereof, can be introduced into an expression vector, such as a viral vector, and the vector can be introduced into appropriate cells in an animal. In such a method, the

10   cell population can be engineered to inducibly or constitutively express active PPARα gene product. In a preferred embodiment, the vector is delivered to the bone marrow, for example as described in Corey *et al.* (*Science 244*:1275-1281 (1989)).

      The invention further provides transgenic nonhuman animals capable of

15   expressing an exogenous reference or variant PPARα gene and/or having one or both alleles of an endogenous PPARα gene inactivated. Expression of an exogenous variant gene is usually achieved by operably linking the gene to a promoter and optionally an enhancer, and microinjecting the construct into a zygote. *See* Hogan *et al.*, "Manipulating the Mouse Embryo, A Laboratory Manual," Cold

20   Spring Harbor Laboratory. Inactivation of endogenous variant genes can be achieved by forming a transgene in which a cloned variant gene is inactivated by insertion of a positive selection marker. *See* Capecchi, *Science* 244, 1288-1292 (1989). The transgene is then introduced into an embryonic stem cell, where it undergoes homologous recombination with an endogenous variant gene. Mice and

25   other rodents are preferred animals. Such animals provide useful drug screening systems.

      In another embodiment, the invention relates to pharmaceutical compositions comprising a reference PPARα gene product. As used herein, a reference PPARα gene product is intended to mean functional gene products which are encoded by the

30   reference allele of the PPARα gene. The invention further relates to the use of compositions (i.e., agonists) which enhance or increase the activity of a peptide comprising the reference PPARα gene product, or a functional portion thereof, for

use in the treatment of disorders of lipid metabolism. The invention also relates to the use of compositions (i.e., antagonists) which reduce or decrease the activity of a peptide comprising the variant PPARα gene product, or a functional portion thereof, for use in the treatment of disorders of lipid metabolism.

5   For instance, the polypeptide or protein, or fragment thereof, of the present invention can be formulated with a physiologically acceptable medium to prepare a pharmaceutical composition. The particular physiological medium may include, but is not limited to, water, buffered saline, polyols (e.g., glycerol, propylene glycol, liquid polyethylene glycol) and dextrose solutions. The optimum concentration of

10   the active ingredient(s) in the chosen medium can be determined empirically, according to procedures well known to those skilled in the art and will depend on the ultimate pharmaceutical formulation desired. Methods of introduction of exogenous peptides at the site of treatment include, but are not limited to, intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, oral and intranasal. Other

15   suitable methods of introduction can also include rechargeable or biodegradable devices and slow release polymeric devices. The pharmaceutical compositions of this invention can also be administered as part of a combinatorial therapy with other agents and treatment regimens.

A non-limiting description of preferred embodiments of the invention follows.

20   EXAMPLES

Methods

Subjects: Two independently recruited cohorts of unrelated adult Caucasians from the Quebec City area and the Saguenay-Lac-St-Jean (SLSJ) region were used to test the hypothesis that genetic variation in PPARα may be associated with the

25   development of dyslipidemia.

Type 2 diabetes case-control SLSJ cohort: All individuals included in this cohort are of French Canadian descent above the age of 18 years and were recruited by the Chicoutimi Hospital Lipid Clinic because they presented a family history of dyslipidemia or diabetes mellitus. These participants had previously received a

30   family code which assured the inclusion in the present study of only one patient per

pedigree in order to control for potential inflation of statistical significance due to lack of independence of observations (12). One hundred and twenty-one unrelated patients who received a new diagnosis of type 2 diabetes following an oral glucose tolerance test (OGTT) (67 men and 54 women; diabetic group), were age and sex-

5   matched (1:1) with a patient having a normal OGTT (control group). Type 2 diabetes was defined according to the World Health Organization criteria as a 2-hour glucose concentration $\geq$ 11.1 mmol/L after a 75g oral glucose absorption, whereas subjects were included in the control group when they had a 2-hour glucose concentration below 7.8 mmol/L (13). Patients diagnosed with type 2 diabetes

10   received dietary counseling according to the American Diabetes Association. Subjects with familial hyperchylomicronemia due to mutations in the lipoprotein lipase gene were excluded from the present study. Subjects were screened for the presence of LDL-receptor gene mutations that cause familial hypercholesterolemia (FH) among French-Canadians. FH screening included two deletions (5 kb and > 15

15   kb) and point mutations W66G, E207K, C646Y, Y268X and R329X (14-16). Waist and hip circumferences were assessed following the procedures recommended by the Airlie Conference (17). Body weight and height were also recorded, and the BMI was calculated in kg/m^2. Medication-free lipoprotein profiles were obtained for each subject enrolled in this study. Total-cholesterol, LDL-cholesterol, HDL-cholesterol,

20   total-apolipoprotein B levels and plasma triglyceride concentrations were measured as previously described (18). Biological and lifestyle variables as well as medical and nutritional histories were obtained through questionnaires and physical exams performed at the Chicoutimi Hospital Lipid Clinic by trained nurses, dieticians and physicians. Smoking habits were classified as follows: 1) patients who never

25   smoked; and 2) patients who ever smoked. Alcohol consumption was treated in two categories: 1) regular drinkers (>5 ounces of absolute alcohol/week); and 2) non-regular drinkers. A subject was considered as hypertensive when diagnosis of essential hypertension had been previously established or when three values of systolic $\geq$ 140 mmHg or diastolic $\geq$ 90 mmHg blood pressure had been recorded in

30   the patient's medical chart. This project has been approved by the Chicoutimi Hospital Ethic Committee.

-30-

Quebec City non-diabetic male cohort: This cohort included 193 men who were recruited from the Quebec City area by solicitation through the media and were selected to cover a wide range of visceral adipose tissue accumulation. All subjects were sedentary, non-smokers and free from metabolic disorders requiring treatment

5   such as diabetes, hypertension and coronary heart disease. A high alcohol consumption was also an exclusion criteria. BMI and waist circumference were measured as described above. Blood samples were obtained after an overnight fast from an antecubital vein. Cholesterol and triglyceride concentrations in plasma and lipoprotein fractions were enzymatically measured on an Analyzer Technicon RA-

10   500 (Bayer Corporation, Tarry Town, NY). VLDL (d<1.006 g/ml) were isolated by ultracentrifugation (19) and the HDL fraction was obtained after precipitation of LDL in the infranatant (d>1.006 g/ml) with heparin and $MnCl_2$ (20). The cholesterol content of HDL_2-chol and HDL_3-chol subfractions prepared by a precipitation method was also determined (21). HDL-apoAI was measured in the

15   infranatant (d>1.006 g/ml) by the rocket immunoelectrophoretic method of Laurell as previously described (22). The serum standards were prepared in the laboratory, calibrated against reference sera from the Centers for Disease Control (Atlanta, GA), lyophilized, and stored at -85°C until use. Coefficients of variation for repeated measurements of HDL-cholesterol and apoAI concentrations were 3.3 and 3.4%,

20   respectively.

Genomic structure of the PPARα gene: In order to design intronic primers for the amplification of each exon, genomic sequences were sought for the intronic regions surrounding all PPARα exons. To do so, the mRNA sequence of PPARα (23) was compared with a contiguous genomic DNA region taken from sequences of three

25   overlapping clones from GenBank (Accession number: Z94161.5, AL049856, ALO78611). Intronic primers were then designed using the Primer 3.0 software available on the Whitehead Institute/MIT Center for Genome Research server (http://www.genome.wi.mit.edu/cgi-bin/primer/primer3.cgi).

PCR amplification of PPARα exons and sequencing: All exons and exon-intron

30   boundaries were amplified from genomic DNA by use of specific primers derived

from the 5' and 3' intronic sequences (Table 1). The annealing temperature for all
primer pairs was 59°C. PCR conditions were as follows: reaction volume was of 50
µl, 1.25 unit AmpliTaq Gold polymerase (Perkin-Elmer Cetus) in the buffer
recommended by the manufacturer; 2.5 mM $MgCl_2$; 0.2 mM dNTPs; primers at a

5   final concentration of 0.5 µM and 100 ng of template genomic DNA. PCR products
were purified with magnetic beads BioMag DNA Sep (PerSeptive Biosystems,
Framingham, MA). Sequencing reactions were performed using BigDye Primer
Cycle sequencing ready reactions -21M13 kit (PE Applied BioSystems, Foster City,
CA) and were analyzed on an ABI 377 automated sequencer (PE Applied

10   BioSystems, Foster City, CA). The gel files were processed using the Sequencing
analysis software (PE Applied BioSystems, Foster City, CA).

**Detection of the L162V by polymerase chain reaction, restriction fragment
length polymorphism (PCR-RFLP):** The L162V mutation is caused by a C→ G
transversion at nucleotide 484 in exon 5. It does not alter any restriction site; thus,

15   mismatch PCR approach was performed with the following primers Ex5.F(2) 5'-
GACTCAAGCTGGTGTATGACAAGT-3' (SEQ ID NO: 3) and Ex5.R-mismatch
5'-CGTTGTGTGACATCCCGACAGAAT-3' (SEQ ID NO: 4). PCR conditions
were as described above. PCR products were digested with HinfI, electrophoresed
on either 12% acrylamide or 4% agarose gel and stained with ethidium bromide.

20   **Statistical analysis:** Allelic frequencies were compared using Chi-square analyses.
Differences between genotypic groups were tested for statistical significance by
using Student's test, and as indicated in Tables 4 and 5, some variables were log_{10}
transformed in order to normalize their distributions. Before performing statistical
analyses on lipoprotein-lipid variables of the type 2 diabetes case-control cohort, 27

25   heterozygous FH subjects were excluded. Analysis of covariance was used to adjust
lipid, lipoprotein and apolipoprotein variables for age alone or for both age and BMI
or age and waist circumference. All statistical analyses were performed using SPSS
package (SPCC Inc.) or JMP statistical package from SAS institute.

-32-

RESULTS

Structure of the human PPARα gene: The structural organization of the gene was
determined by comparing the human PPARα mRNA sequence with genomic
sequences obtained from overlapping clones (Figure 1). All 5' donor and 3' acceptor

5   splice sites were found to obey the gt...ag rule (Table 2). Furthermore, all exon-
intron boundaries were confirmed by direct sequencing of each exon using primers
derived from intronic sequences. The human PPARα gene spans approximately
83.7Kb. As it is the case for the mouse, human PPARα gene mRNA is composed of
8 exons, with a 5' untranslated region encoded by exons 1, 2 and part of exon 3. The

10   coding region of PPARα is comprised within the remaining 6 exons. Intron lengths
vary from 1.4 to 24.8 Kb as indicated in Figure 1.

Identification of polymorphisms in the human PPARα gene: All exons and the
exon-intron splicing boundaries of PPARα were screened by sequencing 12 patients
who have been diagnosed with type 2 diabetes and 2 controls. All the amplified

15   fragments of the PPARα gene showed the expected length, suggesting the absence
of deletions, duplications or rearrangements within these fragments. Two
polymorphisms were discovered within the exons. Based on the predicted amino
acid sequence for this gene, the polymorphism in exon 7 is silent, with a G to A
substitution which does not alter the threonine at codon 253. A new polymorphism

20   was discovered in exon 5, which results in a L162 V missense mutation.
Specifically, the C to G transversion in the first position of codon 162 leads to a
substitution of a valine for a leucine.

Genotype determination of the L162V mutation: A rapid screening test was
developed using the mismatch PCR approach to genotype the L162V

25   polymorphism. Using the primers Ex5R-mismatch and Ex5.F(2) described in the
methods, PCR products from the mutant allele contain an *Hinf*I restriction site that is
abolished in the presence of the C at position 484. After digestion with *Hinf*I, the
PCR product from the normal allele results in a 117 bp fragment, whereas it is
cleaved into 93 bp and 24 bp fragments with the mutant allele. Genotypes obtained

30   by the mismatch PCR approach and by direct sequencing of exon 5 were in perfect

-33-

agreement. Moreover, genotypes obtained by the mismatch PCR approach were repeated for a minimum of 60 randomly chosen individuals, and the reproducibility was 100%.

Association between the L162V missense mutation and lipoprotein-lipid

5   **concentrations:** Chi-square analysis performed independently within the SLSJ control and diabetic groups revealed that confounding factors such as the smoking status, alcohol consumption and the use of beta-blockers or diuretic drugs as well as the prevalence of hypertension were similar between the two PPARα genotypic groups. Furthermore, since patients have been assigned to the diabetic or control

10   group after the OGTT, medication-free lipoprotein-lipid profile as well as fasting blood glucose and insulin levels obtained at the OGTT are shown. Subjects' characteristics according to the L162V genotype are shown in Table 4. Since mutations in the LDL-receptor gene that cause FH have a dramatic effect on the lipoprotein concentrations, heterozygous FH subjects were excluded prior to

15   statistical analyses. Carriers of the rare V162 and L162 homozygotes had similar fasting glucose and insulin levels as well as plasma HDL-cholesterol concentrations, independent of their clinical status: diabetic or control. Although, both diabetics and controls tended to have higher LDL-cholesterol and total-apolipoprotein B levels when they were carriers of the V162 allele, the difference did not reach

20   statistical significance. After adjustments for co-variates such as age and/or BMI, results remained unchanged.

Although not significant, the 9% increase in plasma LDL-cholesterol levels observed among V162 carriers encouraged further investigation of the effect of that missense mutation in a larger cohort. For this purpose, a cohort of 193 non-diabetic

25   males recruited in the greater Quebec City area were screened and for whom a more detailed lipoprotein-lipid profile was obtained. Furthermore, these subjects were free of metabolic disorders requiring treatment such as type 2 diabetes, hypertension or coronary heart disease. In the Quebec City non-diabetic male cohort, the frequency of the rare V162 allele was 6.6% (Table 3). Comparison of the

30   lipoprotein lipid profile between the two genotypic groups showed that as in the diabetes case-control cohort, subjects carrying the rare V162 allele had higher LDL-

cholesterol, LDL-apolipoprotein B and total-apolipoprotein B levels than L162 homozygotes, and these differences reach statistical significance (Table 5). Importantly, these differences remained significant after adjustments for age and BMI or for age and waist circumference.

5   While this invention has been particularly shown and described with references to preferred embodiments thereof, it will be understood by those skilled in the art that various changes in form and details may be made therein without departing from the scope of the invention encompassed by the appended claims.

Table 1: PCR primers for genomic amplification of PPARα exons.

Exons	Oligonucleotides	Product size
Ex.1	PPARαex1.F 5'-**TGTAAAACGACGGCCAGT**CCATCTGGAAAACAGTAAATTAAACC-3' PPARαex1.R 5'-GCATCCAGAGAAACAACCGTAA-3'	169bp
Ex.2	PPARαex2.F 5'-**TGTAAAACGACGGCCAGT**TAATGATAACAGAATTCATCCACCA-3' PPARαex2.R 5'-TCCATTCAAGCTGCTATAACAAAAT-3'	213bp
Ex.3	PPARαex3.F 5'-**TGTAAAACGACGGCCAGT**CGTCAGTCTTACCAATTGTTCCT-3' PPARαex3.R 5'-AAACTTTCTAGGAAACGGCACA-3'	311bp
Ex.4	PPARαex4.F 5'-**TGTAAAACGACGGCCAGT**CGTATGCGAAATCACATCCTC-3' PPARαex4.R 5'-TAAGTAGTTGATGGTGGCGG-3'	300bp
Ex.5	PPARαex5.F 5'-**TGTAAAACGACGGCCAGT**AGTAAAGCAAGTGCGCTGGT-3' PPARαex5.R 5'-AAGGAAGGGGAACTGAGGAA-3'	243bp
Ex.6	PPARαex6.F 5'-**TGTAAAACGACGGCCAGT**CTCACTGCTCATGCCTGTGT-3' PPARαex6.R 5'-CCAAGAGAACCCAGAACAGC-3'	274bp
Ex.7	PPARαex7.F 5'-**TGTAAAACGACGGCCAGT**GCATCCCACATCACCTGAC-3' PPARαex7.R 5'-TCAGTGACATGATACCAGCAGA-3'	530bp
Ex.8	PPARαex8.F 5'-**TGTAAAACGACGGCCAGT**TGATAAGCAGTTCTTGGGTGA-3' PPARgex8.R 5'-ACCATTGAGCATAATTCGCC-3'	566bp

M13 tail inserted into forward primers is indicated in bold.

Table 2: Exon-intron splicing boundaries.

Exon (bp)	Donor....Acceptor	Exon
exon 1 (87)	CAG:gtaa.....acag:TTC	exon 2
exon 2 (84)	GAG:gtac.....ccag:TAG	exon 3
exon 3 (250)	CGG:gtaa.......ccag:ACA	exon 4
exon 4 (161)	AAG:gtag.....acag:GGC	exon 5
exon 5 (139)	ACG:gtag.......ctag:CGA	exon 6
exon 6 (203)	CCA:gtag.....gtag:CCT	exon 7
exon 7 (448)	GAG:gtga.....ctag:ATC	exon 8
exon 8 (480)	TACTGAgttc	

Nucleotides in exons are indicated in uppercase letters, whereas the flanking nucleotides
in introns are in lower case. The exact length in base pairs of the exons is indicated in parenthesis.

Table 3: PPARα allele frequencies.

Population	L162	V162
Study sample # 1 from SLSJ		
- Diabetic patients	213 (88.0%)	29 (12.0%)
- Non-diabetic patients	211 (87.2%)	31 (12.8%)
Study sample # 2, Quebec city non-diabetic men	370 (93.4%)	26 (6.6%)

Number of alleles and percentage in parenthesis.

Table 4: Subjects' characteristics according to the L162V genotype in the study sample # 1 of type 2 diabetic/non-diabetic patients.

Variable	Cases			Controls		
	L/L[1]	L/V[1,2]	p-value	L/L[3]	L/V[4]	p-value
Age, years	53.7±6.7 (85)	53.5±8.0 (26)	0.87	52.5±8.5 (76)	52.5±5.9 (28)	0.99
BMI[*], kg/m^2	29.4±4.2 (85)	28.4±5.2 (26)	0.22	28.3±3.8 (76)	29.2±3.7 (28)	0.22
Waist circumference, cm	98.9±11.7 (80)	94.9±11.6 (26)	0.13	92.5±12.1 (76)	96.2±12.3 (28)	0.16
Fasting glucose[*], mmol/L	6.52±1.95 (86)	6.35±1.16 (26)	0.89	5.19±0.55 (76)	5.15±0.67 (28)	0.71
Fasting Insulin[*], pmol/L	127.8±115.8 (43)	147.0±111.0 (15)	0.44	140.4±116.4 (49)	120.0±91.8 (18)	0.74
Total-cholesterol[*], mmol/L	6.53±1.67 (86)	6.71±1.19 (26)	0.42	6.58±1.43 (76)	6.76±1.59 (28)	0.68
LDL-cholesterol[*], mmol/L	4.15±1.35 (62)	4.53±0.85 (21)	0.11	4.19±0.99 (65)	4.57±1.47 (21)	0.43
HDL-cholesterol[*], mmol/L	0.93±0.32 (81)	0.97±0.27 (26)	0.38	1.04±0.38 (74)	1.02±0.39 (27)	0.85
Triglycerides[*], mmol/L	4.19±3.72 (85)	3.37±2.42 (26)	0.38	2.89±2.39 (76)	3.24±2.90 (28)	0.76
Total apolipoprotein B, g/L	1.20±0.22 (61)	1.29±0.21 (18)	0.12	1.19±0.30 (58)	1.28±0.32 (21)	0.22

[*]Log$_{10}$-transformed variables. Data are means±SD. [1]One homozygote V/V included in the L/V group. Number of subjects is shown in parenthesis. Prior statistical analyses, FH patients were excluded. The male/female ratio was of 48/37, 14/12, 41/35 and 18/10 respectively for groups 1 through 4, group numbers are identified by superscripts.

Table 5: Subjects' characteristics according to the L162V genotype in the study sample # 2 of non-diabetic men.

Variable	L/L	L/V	p-value
Age, years	41.7±8.1 (172)	44.7±7.8 (26)	0.08
BMI, kg/m²	29.9±4.3 (171)	29.1±2.9 (25)	0.36
Waist circumference, cm	101.0±10.7 (171)	99.7±8.9 (26)	0.54
Fasting glucose*, mmol/L	5.41±0.56 (170)	5.33±0.33 (26)	0.55
Fasting Insulin*, pmol/L	109.9±83.7 (170)	85.8±42.5 (26)	0.14
Total-cholesterol, mmol/L	5.21±0.79 (169)	5.50±0.58 (25)	0.08
LDL-cholesterol, mmol/L	3.49±0.74 (169)	3.86±0.56 (25)	0.02
HDL-cholesterol*, mmol/L	0.93±0.20 (169)	0.91±0.17 (25)	0.70
HDL₂-cholesterol, mmol/L	0.26±0.16 (166)	0.27±0.17 (25)	0.78
Triglycerides*, mmol/L	2.17±1.16 (169)	1.98±0.80 (25)	0.66
Total-apolipoprotein B, g/L	1.07±0.24 (166)	1.18±0.16 (25)	0.02
LDL-apolipoprotein B, g/L	0.94±0.20 (166)	1.05±0.13 (25)	0.01

* Log₁₀-transformed variables. Data are means±SD. Number of subjects is shown in parenthesis.

REFERENCES

1. Schoonjans K, Martin G, Staels B, Auwerx J. Peroxisome proliferator-activated receptors, orphans with ligands and functions. Curr.Opin.Lipidol. 1997;8:159-166

2. Schoonjans K, Staels B, Auwerx J. The peroxisome proliferator activated receptors (PPARS) and their effects on lipid metabolism and adipocyte differentiation. Biochim Biophys Acta 1996;1302:93-109

3. Schoonjans K, Staels B, Auwerx J. Role of the peroxisome proliferator-activated receptor (PPAR) in mediating the effects of fibrates and fatty acids on gene expression. J Lipid Res 1996;37:907-925

4. Vidal-Puig AJ, Considine RV, Jimenez-Linan M, et al. Peroxisome proliferator-activated receptor gene expression in human tissues. Effects of obesity, weight loss, and regulation by insulin and glucocorticoids. J Clin Invest 1997;99:2416-2422

5. Brun RP, Tontonoz P, Forman BM, et al. Differential activation of adipogenesis by multiple PPAR isoforms. Genes Dev. 1996;10:974-984

6. Isseman I, Green S. Activation of a member of the steroid hormone receptor superfamily by peroxisome proliferators. Nature 1990;347:645-650

7. Auboeuf D, Rieusset J, Fajas L, et al. Tissue distribution and quantification of the expression of mRNAs of peroxisome proliferator-activated receptors and liver X receptor-alpha in humans: no alteration in adipose tissue of obese and NIDDM patients. Diabetes 1997;46:1319-1327

8. Kliewer SA, Sundseth SS, Jones SA, et al. Fatty acids and eicosanoids regulate gene expression through direct interactions with peroxisome proliferator-activated receptors alpha and gamma. Proc.Natl.Acad.Sci.U.S.A 1997;94:4318-4323

9. Inoue I, Noji S, Shen MZ, Takahashi K, Katayama S. The peroxisome proliferator-activated receptor alpha (PPAR alpha) regulates the plasma thiobarbituric acid-reactive substance (TBARS) level. Biochem.Biophys.Res.Commun. 1997;237:606-610

10. Horrobin DF. Abnormal membrane concentrations of 20 and 22-carbon essential fatty acids: a common link between risk factors and coronary and peripheral vascular disease? Prostaglandins Leukot.Essent.Fatty Acids. 1995;53:385-396

11. Reaven GM. Syndrome X: 6 years later. J.Intern.Med.Suppl. 1994;736:13-22:13-22

12. Gaudet D, Arsenault S, Belanger C, et al. Procedure to protect confidentiality of familial data in community genetics and genomic research [In Process Citation]. Clin.Genet. 1999;55:259-264

13.   Report on the Expert Committee on the diagnosis and classification of diabetes mellitus.
       Diabetes Care 1997;20:1183-1197

14.   Couture P, Vohl MC, Gagné C, et al. Identification of three mutations in the low-density
       lipoprotein receptor gene causing familial hypercholesterolemia among French-
       Canadians. Hum.Mutat. 1997;suppl 1:s226-s231

15.   Ma Y, Bétard C, Roy M, Davignon J, Kessling A. Identification of a second "French
       Canadian" LDL receptor gene deletion and development of a rapid method to detect
       both deletions. Clinical Genetics 1989;36:219-228

16.   Vohl MC, Couture P, Moorjani S, et al. Rapid restriction fragment analysis for screening
       four point mutations of the low-density lipoprotein receptor gene in French
       Canadians. Hum Mutat. 1995;6:243-246

17.   Standardization of anthropometric measurements. In: *The Airlie (VA) Consensus
       Conference.*, edited by Lohman, T., Roche, A., Martorel, R. Human Kinetics
       Publishers, 1988;39-80.

18.   Gaudet D, Vohl MC, Julien P, et al. Relative contribution of low-density lipoprotein
       receptor and lipoprotein lipase gene mutations to angiographically assessed
       coronary artery disease among french Canadians. Am J Cardiol 1998;82:299-305

19.   Havel RJ, Eder HA, Bragdon JH. The distribution and chemical composition of
       ultracentrifugally separated lipoproteins in human serum. J Clin Invest
       1955;34:1345-1354

20.   Burstein M, Samaille J. Sur un dosage rapide du cholesterol lié aux alpha et aux béta
       lipoproteines du sérum. Clin Chem Acta 1960;5:309

21.   Gidez LI, Miller GJ, Burnstein M, Slagle S, Eder HA. Separation and quantitation of
       subclasses of human plasma high density lipoproteins by a simple precipitation
       procedure. J.Lipid Res. 1982;23:1206-1223

22.   Laurell CB. Quantitative estimation of proteins by electrophoresis in agarose gel
       containing antibodies. Anal Biochem 1966;15:42

23.   Mukherjee R, Jow L, Noonan D, McDonnell DP. Human and rat peroxisome proliferator
       activated receptors (PPARs) demonstrate similar tissue distribution but different
       responsiveness to PPAR activators. J Steroid Biochem.Mol.Biol 1994;51:157-166

24.   Sher T, Yi HF, McBride OW, Gonzalez FJ. cDNA cloning, chromosomal mapping, and
       functional characterization of the human peroxisome proliferator activated receptor.
       Biochem 1993;32:5598-5604

25.   Gearing KL, Crickmore A, Gustafsson JA. Structure of the mouse peroxisome
       proliferator activated receptor alpha gene. Biochem.Biophys.Res.Commun.
       1994;199:255-263

26. Tugwood JD, Aldridge TC, Lambe KG, Macdonald N, Woodyatt NJ. Peroxisome proliferator-activated receptors: structures and function. Ann.N.Y.Acad.Sci. 1996;804:252-65:252-265

27. Holden PR, Tugwood JD. Peroxisome proliferator-activated receptor alpha: role in rodent liver cancer and species differences. J.Mol.Endocrinol. 1999;22:1-8

28. Castelli WP, Garrison RJ, Wilson PWF, Abbot RD, Kalousdian S, Kannel WB. Incidence of coronary heart disease and lipoprotein cholesterol levels: the Framingham study. JAMA 1986;256:2835-2838

29. Castelli WP, Anderson K, Wilson PW, Levy D. Lipids and risk of coronary heart disease. The Framingham Study. Ann.Epidemiol. 1992;2:23-28

30. Lamarche B, Tchernof A, Mauriege P, et al. Fasting insulin and apolipoprotein B levels and low-density lipoprotein particle size as risk factors for ischemic heart disease. JAMA 1998;279:1955-1961

31. Bolibar I, Thompson SG, von Eckardstein A, Sandkamp M, Assmann G. Dose-response relationships of serum lipid measurements with the extent of coronary stenosis. Strong, independent, and comprehensive. ECAT Angina Pectoris Study Group. Arterioscler.Thromb.Vasc.Biol. 1995;15:1035-1042

32. Djouadi F, Weinheimer CJ, Saffitz JE, et al. A gender-related defect in lipid metabolism and glucose homeostasis in peroxisome proliferator- activated receptor alpha-deficient mice. J Clin.Invest. 1998;102:1083-1091

33. Peters JM, Hennuyer N, Staels B, et al. Alterations in lipoprotein metabolism in peroxisome proliferator- activated receptor alpha-deficient mice. J Biol.Chem. 1997;272:27307-27312

34. Lamb RG, Koch JC, Bush SR. An enzymatic explanation of the differential effects of oleate and gemfibrozil on cultured hepatocyte triacylglycerol and phosphatidylcholine biosynthesis and secretion. Biochim.Biophys.Acta 1993;1165:299-305

35. Ruotolo G, Ericsson CG, Tettamanti C, et al. Treatment effects on serum lipoprotein lipids, apolipoproteins and low density lipoprotein particle size and relationships of lipoprotein variables to progression of coronary artery disease in the Bezafibrate Coronary Atherosclerosis Intervention Trial (BECAIT). J Am Coll.Cardiol. 1998;32:1648-1656

36. Ericsson CG. Results of the Bezafibrate Coronary Atherosclerosis Intervention Trial (BECAIT) and an update on trials now in progress. Eur.Heart J 1998;19 Suppl H:H37-41:H37-H41

-43-

37. de Faire U, Ericsson CG, Grip L, Nilsson J, Svane B, Hamsten A. Retardation of coronary atherosclerosis: the Bezafibrate Coronary Atherosclerosis Intervention Trial (BECAIT) and other angiographic trials. Cardiovasc.Drugs Ther. 1997;11 Suppl 1:257-63:257-263

38. Bell AR, Savory R, Horley NJ, et al. Molecular basis of non-responsiveness to peroxisome proliferators: the guinea-pig PPARalpha is functional and mediates peroxisome proliferator- induced hypolipidaemia. Biochem.J 1998;332:689-693

39. Gottlicher M, Widmark E, Li Q, Gustafsson JA. Fatty acids activate a chimera of the clofibric acid-activated receptor and the glucocorticoid receptor. Proc.Natl.Acad.Sci.U.S.A. 1992;89:4653-4657

-44-

CLAIMS

What is claimed is:

1.   A method for predicting the likelihood that an individual will have a disorder
      of lipid metabolism, comprising the steps of obtaining a DNA sample from an
5   individual to be assessed and determining the nucleotide present at the first
      position of codon 162 of the PPARα gene, wherein the presence of a C at this
      position indicates that the individual has a lower likelihood of having a
      disorder of lipid metabolism than an individual having a G at that position, or
      a greater likelihood of having less severe symptomology.

10   2.   A method for predicting the likelihood that an individual will have a disorder
      of lipid metabolism, comprising the steps of obtaining a DNA sample from an
      individual to be assessed and determining the nucleotide present at the first
      position of codon 162 of the PPARα gene, wherein the presence of a G at this
      position indicates that the individual has a greater likelihood of having a
15   disorder of lipid metabolism than an individual having a C at that position, or
      a greater likelihood of having more severe symptomology.

3.   A method according to Claim 1 or 2, wherein the disorder is selected from the
      group consisting of abnormal lipoprotein-lipid variability, abnormal LDL-
      cholesterol levels, abnormal plasma total-apolipoprotein B levels,
20   atherosclerosis, hyperapobetalipoproteinemia and coronary heart disease.

4.   A method for predicting the likelihood that an individual will have a disorder
      associated with a G at nucleotide position 696 of the PPARα gene comprising
      the steps of obtaining a DNA sample from an individual to be assessed and
      determining the nucleotide present at position 696 of the PPARα gene,
25   wherein the presence of a G at this position indicates that the individual has a
      greater likelihood of having a disorder associated therewith than an individual

-45-

having a C at that position, or a greater likelihood of having more severe symptomology.

5. A method for predicting the likelihood that an individual will have a disorder associated with a C at nucleotide position 696 of the PPARα gene comprising the steps of obtaining a DNA sample from an individual to be assessed and determining the nucleotide present at position 696 of the PPARα gene, wherein the presence of a C at this position indicates that the individual has a greater likelihood of having a disorder associated therewith than an individual having a G at that position, or a greater likelihood of having more severe symptomology.

6. A method for predicting the likelihood that an individual will have a disorder associated with an A at nucleotide position 971 of the PPARα gene comprising the steps of obtaining a DNA sample from an individual to be assessed and determining the nucleotide present at position 971 of the PPARα gene, wherein the presence of an A at this position indicates that the individual has a greater likelihood of having a disorder associated therewith than an individual having a G at that position, or a greater likelihood of having more severe symptomology.

7. A method for predicting the likelihood that an individual will have a disorder associated with a G at nucleotide position 971 of the PPARα gene comprising the steps of obtaining a DNA sample from an individual to be assessed and determining the nucleotide present at position 971 of the PPARα gene, wherein the presence of a G at this position indicates that the individual has a greater likelihood of having a disorder associated therewith than an individual having a A at that position, or a greater likelihood of having more severe symptomology.

8. A method for predicting the likelihood that an individual will have a disorder of lipid metabolism, comprising the steps of obtaining a biological sample

-46-

comprising the PPARα protein or relevant portion thereof from an individual

to be assessed and determining the amino acid present at amino acid position

162 of the PPARα protein, wherein the presence of a leucine at this position

indicates that the individual has a lower likelihood of having a disorder of

5   lipid metabolism than an individual having a valine at that position, or a

greater likelihood of having less severe symptomology.

9.   A method for predicting the likelihood that an individual will have a disorder

of lipid metabolism, comprising the steps of obtaining a.biological sample

comprising the PPARα protein or relevant portion thereof from an individual

10   to be assessed and determining the amino acid present at amino acid position

162 of the PPARα protein, wherein the presence of a valine at this position

indicates that the individual has a greater likelihood of having a disorder of

lipid metabolism than an individual having a leucine at that position, or a

greater likelihood of having more severe symptomology.

15   10.   A method for predicting the likelihood that an individual will have a disorder

associated with a valine at amino acid position 162 of the PPARα protein

comprising the steps of obtaining a biological sample comprising the PPARα

protein or relevant portion thereof from an individual to be assessed and

determining the amino acid present at amino acid position 162 of the PPARα

20   protein, wherein the presence of a valine at this position indicates that the

individual has a greater likelihood of having a disorder associated therewith

than an individual having a leucine at that position, or a greater likelihood of

having more severe symptomology.

11.   A method for predicting the likelihood that an individual will have a disorder

25   associated with a leucine at amino acid position 162 of the PPARα protein,

comprising the steps of obtaining a biological sample comprising the PPARα

protein or relevant portion thereof from an individual to be assessed and

determining the amino acid present at amino acid position 162 of the PPARα

protein, wherein the presence of a leucine at this position indicates that the

-47-

individual has a greater likelihood of having a disorder associated therewith than an individual having a valine at that position, or a greater likelihood of having more severe symptomology.

12.   A pharmaceutical composition comprising a peptide comprising a functional
5   portion of PPARα reference gene product for use in the treatment of disorders
      of lipid metabolism.

13.   A method of treating a disorder of lipid metabolism, comprising administering
      to an individual in need thereof a pharmaceutical composition comprising a
      peptide comprising a functional portion of PPARα reference gene product.

10   14.   A pharmaceutical composition comprising a nucleic acid molecule encoding a
      peptide comprising a functional portion of PPARα reference gene product for
      use in the treatment of disorders of lipid metabolism.

15.   A method of treating a disorder of lipid metabolism, comprising administering
      to an individual in need thereof a pharmaceutical composition comprising a
15   nucleic acid molecule encoding a peptide comprising a functional portion of
      PPARα reference gene product.

16.   A method of treating a disorder of lipid metabolism, comprising administering
      to an individual in need thereof a pharmaceutical composition comprising an
      agonist of a peptide comprising a functional portion of PPARα reference gene
20   product.

17.   An isolated nucleic acid molecule comprising all or a portion of the variant
      allele of PPARα.

18.   An isolated nucleic acid molecule according to Claim 17, wherein wildtype
      PPARα is exemplified by SEQ ID NO: 1.

-48-

19. An isolated gene product which is encoded by a nucleic acid molecule according to Claim 17.

20. An isolated polypeptide comprising all or a portion of the variant amino acid sequence of PPARα.

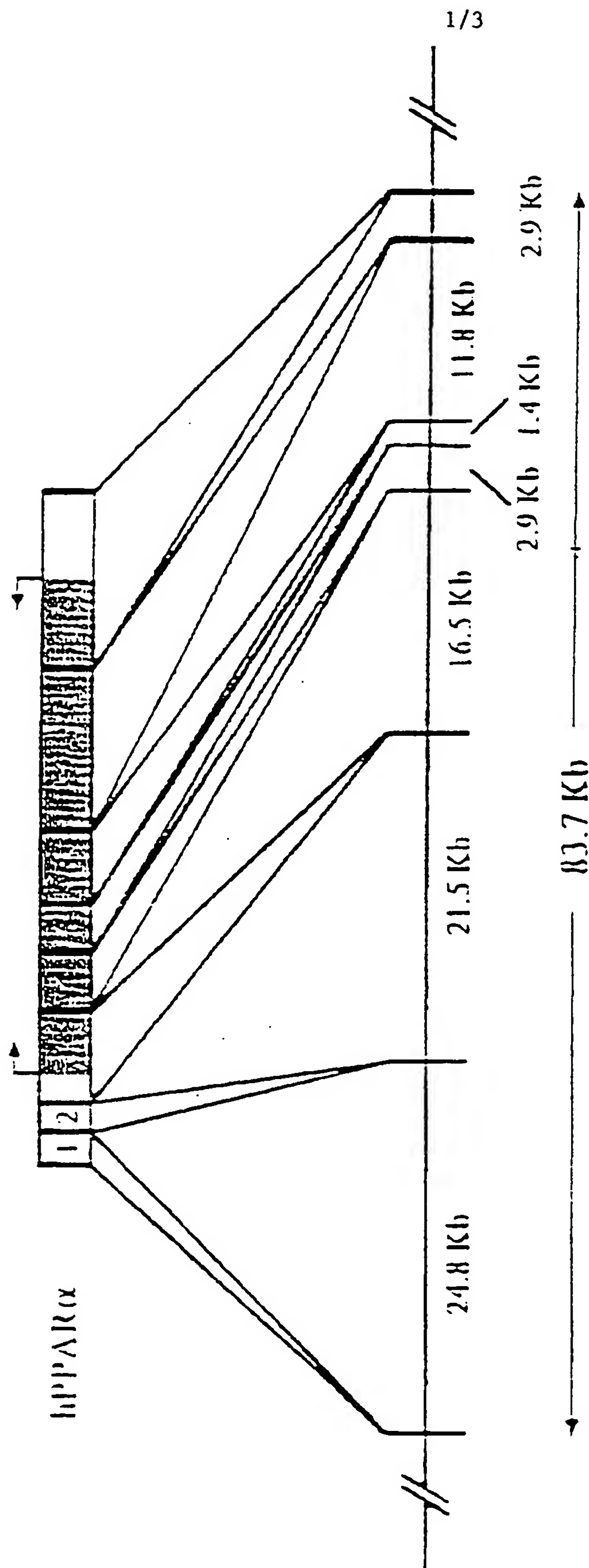5 21. An isolated polypeptide according to Claim 20, wherein wildtype PPARα is exemplified by SEQ ID NO: 2.

Figure 1

```
ggcccaggct gaagctcagg gccctgtctg ctctgtggac tcaacagttt gtggcaagac
aagctcagaa ctgagaagct gtcaccacag ttctggaggc tgggaagttc aagatcaaag
tgccagcaga ttcagtgtca tgtgaggacg tgcttcctgc ttcatagata agagcttgga
gctcggcgca caaccagcac catctggtcg cgatggtgga cacgaaagc ccactctgcc
ccctctcccc actcgaggcc ggcgatctag agagcccgtt atctgaagag ttcctgcaag
aaatgggaaa catccaagag atttcgcaat ccatcggcga ggatagttct ggaagctttg
gctttacgga ataccagtat ttaggaagct gtcctggctc agatggctcg gtcatcacgg
acacgctttc accagcttcg agcccctcct cggtgactta tcctgtggtc cccggcagcg
tggacgagtc tcccagtgga gcattgaaca tcgaatgtag aatctgcggg gacaaggcct
caggctatca ttacggagtc cacgcgtgtg aaggctgcaa gggcttcttt cggcgaacga
ttcgactcaa gctggtgtat gacaagtgcg accgcagctg caagatccag aaaaagaaca
gaaacaaatg ccagtattgt cgatttcaca agtgcctttc tgtcggatg tcacacaacg
cgattcgttt tggacgaatg ccaagatctg agaaagcaaa actgaaagca gaaattctta
cctgtgaaca tgacatagaa gattctgaaa ctgcagatct caaatctctg gccaagacaa
tctacgaggc ctacttgaag aacttcaaca tgaacaaggt caaagcccgg gtcatcctct
caggaaaggc cagtaacaat ccacctttg tcatacatga tatggagaca ctgtgtatgg
ctgagaagac gctggtggcc aagctggtgg ccaatggcat ccagaacaag gaggcggagg
tccgcatctt tcactgctgc cagtgcacgt cagtggagac cgtcacggac ctcacggaat
tcgccaaggc catcccaggc ttcgcaaact tggacctgaa cgatcaagtg acattgctaa
aatacggagt ttatgaggcc atattcgcca tgctgtcttc tgtgatgaac aaagacggga
tgctggtagc gtatggaaat gggtttataa ctcgtgaatt cctaaaaagc ctaaggaaac
cgttctgtga tatcatggaa cccaagtttg, attttgccat gaagttcaat gcactggaac
tggatgacag tgatatctcc cttttgtgg ctgctatcat ttgctgtgga gatcgtcctg
gccttctaaa cgtaggacac attgaaaaaa tgcaggaggg tattgtacat gtgctcagac
tccacctgca gagcaaccac ccggacgata tctttctctt cccaaaactt cttcaaaaaa
tggcagacct ccggcagctg gtgacggagc atgcgcagcc ggtgcagatc atcaagaaga
cggagtcgga tgctgcgctg caccgctac tgcaggagat ctacaggcac atgtactgag
ttccttcaga tcagccacac ctttccagg agttctgaag ctgacagcac tacaaaggag
acggggagc agcacgattt tgcacaaata tccaccactt taaccttaga gcttggacag
tctgagctgt aggtaaccgg catattattc catatctttg tttttaaccag tacttctaag
agcatagaac tcaaatgctg ggggaggtgg ctaatcttag gactgggaag (SEQ ID NO: 1)
```

FIGURE 2

MVDTESPLCPLSPLEAGDLESPLSEEFLQEMGNIQEISQSIGEDSSGSFGFTEYQYLGSCPGSDGSVITDTLSPA
SSPSSVTYPVVPGSVDESPSGALNIECRICGDKASGYHYGVHACEGCKGFFRRTIRLKLVYDKCDRSCKIQK
KNRNKCQYCRFHK CLSVGMSHNAIRFGRMPRSEKAKLKAEILTCEHDIEDSETADLKSLAKRIYEAYLKNF
NMNKVKARVILSGKASNNPPFVIHDMETLCMAEKTLVAKLVANGIQNKEAEVRIFHCCQCTSVETVTELTE
FAKAIPGFANLDLNDQVTLLKYGVYEAIFAMLSSVMNKDGMLVAYGNGFITREFLKSLRKPFCDIMEPKFD
FAMKFNALELDDSDISLFVAAIICCGDRPGLLNVGHIEKMQEGIVHVLRLHLQSNHPDDIFLFPKLLQKMAD
LRQLVTEHAQLVQIIKKT ESDAALHPLLQEIYRDMY (SEQ ID NO: 2)

FIGURE 3